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Invention:

EIAV VACCINE AND DIAGNOSTIC

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EIA VACCINE AND DIAGNOSTIC

BACKGROUND OF THE INVENTION

Related Application:

This application is a continuation-in-part of US application serial number 10/180,626, filed on June 26, 2002, which is a divisional of US application serial number 09/658,547, filed on September 9, 2000, now US Patent No. 6,585,978.

Field of the Invention:

This invention pertains to an EIA vaccine and/or construct, which provides immunity from disease and/or infection with EIAV, which vaccine, in various embodiments, allows diagnostic differentiation between vaccinated and non-vaccinated, but exposed or diseased mammals. In various embodiments, this invention pertains to a vaccine comprising an EIAV wherein an accessory gene has been made nonfunctional and wherein said nonfunctional accessory gene still allows the EIAV to replicate in tissue culture.

Brief Description of the Prior Art:

The equine infectious anemia virus is a member of the lentivirus subfamily of retroviruses and causes persistent infection and chronic disease in horses worldwide. As such, it is closely related to human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV). As with HIV and SIV, disease caused by EIAV is spread by blood transmission. With EIAV, the blood transmission most often occurs by biting flies and other insects carrying virus particles from one horse to another. The first cycle of disease (clinical episode or first febrile episode) in an infected horse usually occurs within 42 days after transmission of

the virus. This first cycle is usually characterized by the acute stage of EIA and manifested by pyrexia, thrombocytopenia, anorexia, depression and high plasma viremia levels. Anemia is not usually detected at this stage. Resolution of this first febrile episode is normally observed after 1 to 5 days and occurs concomitantly with a dramatic drop in the amount of plasma-associated virus. Following the acute stage, some animals may remain clinically normal, while others go on to experience multiple bouts of illness in which severe anemia may accompany pyrexia, thrombocytopenia, edema, and dramatic weight loss, and death. Nucleotide sequence data has revealed a high mutation rate of this lentivirus genome during persistent infection (Payne et al, Virology, 1987: 161, p. 321-331) incorporated herein by reference. It is generally known that multiple isolates from the field demonstrate similar genomic differences indicating that EIAV, as HIV and FIV, undergoes a continuing mutation process within its various hosts. It is generally thought that neutralizing antibodies aid in the selection of new antigenic virus variants (mutations) during persistent infections. In infections with EIAV, serologically distinct variants emerge possibly through immune selection pressure operating on random viral genome mutations. It is proposed that horses that show no further clinical signs of disease have developed a mature immune response that can contain the virus and its immunologically-recognized mutants.

The disease is significant because horses that demonstrate exposure to EIAV via testing for antibodies in the blood (Coggins Test or similar anti-p26 antibody detecting test) are either required to be destroyed or strictly quarantined. Because of the Coggins Test and its broad use in the world, especially in testing all performance horses that are

transferred into and out of the United States, it is critical that vaccinated equines be able to be differentiated from infected equines.

The genetic organization of EIAV, as with HIV, SIV and FIV contains only three accessory genes (*S1, S2* and *S3*), in addition to the *gag, pol* and *env* genes common to all retroviruses. The *S1* open reading frame (ORF) encodes the viral Tat protein, a transcription *trans* activator that acts on the viral long-terminal-repeat (LTR) promoter element to stimulate expression of all viral genes. The *S3* ORF encodes a Rev protein, a post-transcriptional activator that acts by interacting with its target RNA sequence, named the Rev-responsive element (RRE), to regulate viral structural gene expression. The *S2* gene is located in the *pol-env* intergenic region immediately following the second exon of Tat and overlapping the amino terminus of the Env protein (see Figures 1, 2a and 2b). It encodes a 65-amino-acid protein with a calculated molecular mass of 7.2 kDa, which is in good agreement with the size of an *in vitro* translation product. *S2* appears to be synthesized in the late phase of the viral replication cycle by ribosomal leaky scanning of a tricistronic mRNA encidong Tat, *S2* protein, and *Env,* respectively. The ORF coding for the *S2* protein of EIAV is highly conserved in all published EIAV sequences and contains three potential functional motifs (Figures 2a): GLFG (putative nucleoporin motif), PXXP (putative SH3 domain binding motif) and RRKQETKK (putative nuclear localization sequence). Antibodies to *S2* protein can be found in sera from experimentally and naturally infected horses, indicating that *S2* is expressed during EIAV replication in vivo. These observations suggest that *S2* is likely to perform an important role in the virus life cycle. A discussion of the function of *S2* is found in Li et al (J. Virol., Oct. 1998, p 8344-8348), incorporated herein by reference.

A second interesting gene contained within the lentivirus group codes for dUTPase. This enzyme catalyzes the conversion of dUTP to dUMP and pp_i. The gene encoding the dUTPase has been mapped within the *pol* gene for EIAV and FIV. The lentivirus dUTPase gene has been designated *DU*. Studies with *DU* deletion mutants (*ΔDU*) of EIAV and FIV show that this enzyme is not required for replication of the viruses in fetal equine kidney cells or Crandell cells. However, efficient replication of the EIAV or FIV in monocyte/macrophage cells (typical replication host cell) does require *DU*. The differences indicated have been described in detail in a publication by Lichtenstein et al (J. Virol., May 1995, p 2881-2888), incorporated herein by reference.

Envelope proteins (*env*) are thought to be required for protection from disease and, perhaps, protection from infection. By protection from disease is meant that a mammal exposed to the virus, does not demonstrate clinical signs (fever, lethargy, anemia, etc.) but does carry particles associated with the viral RNA genome (shortened herein to viral particles) in its blood, said particles being detectable by a reverse transcriptase polymerase chain reaction test (RT-PCR). By protection from infection is meant that a mammal exposed to the virus does not demonstrate clinical signs nor does its blood contain RT-PCR-detectable virus particles as described above. The major envelope proteins of EIAV are gp90 and gp45. These are proposed as the protective antigens of EIAV. By the term protective antigens is meant antigens from EIAV that produce either protection from disease or protection from infection as indicated above.

It would seem obvious to prepare a vaccine by purifying out the *env* proteins, especially gp90 and gp45. Indeed, preparation of vaccines comprising gp90 and gp45 has been attempted with essentially no success. Issel et al (J. Virol. June 1992, p 3398-

3408) report that a gp90/gp45 vaccine protected ponies from infection caused by homologous EIAV (the subunits were derived from the same EIAV strain as was used for challenge), however, these subunits did not protect ponies from either disease or infection when challenged with a heterologous EIAV strain. In fact, the latter produced enhanced disease signs. The subunit enhancement corroborates findings with SIV and FIV subunit vaccines that appear to enhance disease post challenge. These authors conclude that perfecting a subunit vaccine for lentiviruses (e.g., HIV, SIV, EIAV and FIV) poses a significant challenge because of the subunit enhancement effect.

Issel, et al (J. Virol., June 1992, pp 3398-3408) report the prevention of infection by a high-dose whole-virus EIA vaccine. However, vaccination of horses with this vaccine produces horses that are Coggins Test positive (anti-p26 antibody positive) and there is no practical method to demonstrate the difference between vaccinated and infected equines. Due to the previously-mentioned eradication program in effect in the U.S., a whole-virus vaccine is not feasible.

Since there has been no effective and safe method for immunizing mammals against lentiviral diseases, particularly equines against EIAV and since EIAV is such a wide-spread and significant disease world-wide, there remains a long-felt need to prepare such a vaccine.

SUMMARY OF THE INVENTION

The vaccine of this invention provides the first successful vaccine that effectively and safely immunizes mammals, especially equids, from disease and/or infection caused by EIAV wherein vaccinated mammals can be differentiated from wild-type EIA infected mammals.

Embodiments of this invention describe a vaccine for effectively and safely immunizing mammals, especially equids, from disease caused by EIAV, said vaccine comprising a gene-mutated EIAV wherein said virus lacks the ability to express the mutated gene protein *in vivo* and wherein said lack of expression can be used to differentiate vaccinated from non-vaccinated or infected mammals.

Encompassed within embodiments of this invention is an EIAV wherein said virus contains a mutation in a gene that allows replication of the virus *in vitro* such that large-scale production can be accomplished.

Also encompassed within embodiments of this invention is an EIAV wherein said virus contains a mutation of the *S2* gene or portions thereof ($\Delta S2$), a mutation in the *DU gene* (ΔDU) or a portion thereof, a mutation in a regulatory gene that inhibits expression of the *S2* or *DU* genes or a combination of types of said mutations ($\Delta S2\Delta DU$). It is expected that further mutations can be made such that the EIAV in the vaccine contains multiple mutations in multiple genes including the $\Delta S2$, ΔDU or both.

It is within the scope of embodiments of this invention that a diagnostic test can be used to differentiate vaccinated equines from non-vaccinated and/or infected equines by measuring the presence or absence of antibodies to the *S2* protein, to the *DU* protein or to both proteins. Also, a PCR-based diagnostic test could be used to detect the presence or absence of the *S2* and/or *DU* genes or gene sequences in the equine and, thus, detect whether an equine had been infected with EIAV or vaccinated with the composition of this invention.

Finally, it is expected that said mutated regions could serve as points for insertion of foreign genes or gene sequences and that said $\Delta S2$ or ΔDU or combination thereof

with a foreign gene insert could be useful as a vector for vaccination against diseases of mammals other than EIA. Preferably, the insertion would be placed into the Δ*DU* region.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of replication competent EIAV including the location of the accessory genes of $EIAV_{UK}$.

Figure 2a is a schematic representation of the EIAV *S2* gene and mutant clones derived from $EIAV_{UK}$.

Figure 2b is a schematic representation of the Wild-type EIAV *S2* gene compared with the EIAV2M/X ($EIAV_{UK}$ Δ*S2*) gene.

Figure 3a is a circular map of biological proviral clone $EIAV_{PR}$.

Figure 3b is a circular map of molecular infectious clone $EIAV_{UK}$.

Figure 3c is a circular map of mutant $EIAV_{UK}$Δ*S2*.

Figure 3d is a circular map of mutant $EIAV_{PR}$Δ*S2*.

Figure 3e is a circular map of mutant $EIAV_{UK}$Δ*DU*Δ*S2*.

Figure 4 are graphs demonstrating the *in vitro* replication of EIA virus mutant clones.

Figure 5 is a schematic representation of the *DU* gene location and construction of EIAVΔ*DU*.

Figure 6 is a representation of the nucleotide regions considered for nucleotide deletions by their respective locations in the viral genome.

Figure 7 is a representation of the 14 (D14) and 25 (D25) base pair nucleotide deletions as compared to the $EIAV_{UK}$ΔS2 (ΔS2). Alignment gaps (-) are introduced for better visualization.

Figure 8 is a graph demonstrating the *in vitro* replication in ED cells of the EIA virus and mutant clones.

Figure 9 is a representation of the 6 (D6) and 9 (D9) base pair nucleotide deletions as compared to the $EIAV_{UK}\Delta S2$ ($\Delta S2$). Alignment gaps (-) are introduced for better visualization.

Figure 10 is a graph demonstrating the *in vitro* replication in ED cells of the EIA virus and mutant clones.

Figure 11 is a graph demonstrating the *in vitro* replication in equine macrophage of the EIA virus and mutant clones.

DETAILED DESCRIPTION OF THE INVENTION

This invention encompasses a composition for effectively and safely immunizing mammals from disease caused by EIAV, said composition comprising a gene-mutated EIAV wherein said virus lacks the ability to express the mutated gene protein in vivo and wherein said lack of expression can be used to differentiate vaccinated from non-vaccinated or infected mammals. It is contemplated that any gene could be mutated from any EIAV as long as the mutated gene would allow large-scale production of EIAV or EIAV particles. It is further contemplated that more than one gene from EIAV could be mutated. It is also understood that the composition of the present invention produces a mature immune response capable of protecting equines from disease caused by heterologous as well as homologous EIAV strains.

By gene-mutated is meant that one or more deletions or insertions are made in a gene of EIAV which makes the gene non-functional and thus, differentiating between the gene-mutated virus and wild-type virus. The gene mutation can either be produced

biologically, by passaging the virus through cells, cell lines or animals until it becomes non-infective and a gene-mutated virus is produced, or molecularly (produced by recombinant techniques). By non-functional is meant that the gene does not express its protein or product at all or is not expressing its normal gene product. By a differentiating gene is meant that the gene product is normally expressed by wild-type EIAV and antibodies to the gene product are found in infected horses but not in horses vaccinated with the vaccine compositions of the present invention. By large-scale production is meant that the gene-mutated EIAV can be grown or replicated *in vitro* such that large quantities (e.g., >1 liter, preferably greater than 10 liters) can be produced for vaccine manufacture. Such large-scale production is accomplished if a virus or virus construct can be produced that is stable, withstands concentration and/or purification, if necessary, is stable to adjuvants and storage as a vaccine for up to 18 months. By deletion is meant that all or a portion of a gene of EIAV is removed thus causing the gene to become non-functional. By insertion is meant that all or part of another gene or a sequence of nucleotides (e.g., a stop codon) is inserted into a gene causing it to express a different protein (e.g., one expressed by the inserted gene) and become non-functional for the normally-expressed protein or become non-functional by the insertion of a stop codon. Encompassed by this invention is an EIAV wherein said virus contains a mutation of the *S2* gene or portions thereof (Δ*S2*), a mutation in the *DU* gene (Δ*DU*) or portions thereof, a mutation in a regulatory gene that controls expression of *S2* or *DU,* or a combination of such mutations affecting both genes (Δ*S2*Δ*DU*). Further mutations can be made such that the EIAV in the vaccine contains multiple mutations in multiple genes, including the ΔS2, ΔDU or both. Said mutations would produce a non-functional *S2* and/or *DU* gene.

Illustratively, it has been demonstrated that placing a stop codon into the *S2* gene, replacing amino acid G^5 produced a non-functional *S2* gene. Additionally, it has been demonstrated that changing the *S2* gene's M^{16} to T and replacing the G^5 and G^{18} with stop codons produced a non-functional *S2* gene. Finally, it has been demonstrated that deletion of the initial 5 nucleotides of *S2* produced a non-functional *S2* gene. Therefore, mutations in the *S2* gene have produced EIAV with non-functional *S2* genes. The following is an illustration, but non-limiting description of how to produce the above mutations. Two adjacent fragments were amplified by PCR spanning the whole *S2* gene. One of the two resultant PCR products carried the specific substitution or deletion mutations incorporated into a PCR primer. The flanking PCR products were phosphorylated, ligated, and then used as a template for a second round of PCR with the outer primer pair. The final full-length PCR product was digested with NcoI and Blp1 (previously classified as Bpu11021), cloned into $EIAV_{UK}$ previously digested with NcoI and Blp1. All plasmid clones were sequenced to verify introduced mutations to ensure the integrity of the PCR-amplified sequence. It is important to note that the above-identified mutant EIAV clones replicated well *in vitro*, especially in fetal equine kidney cells (FEK), in equine blood monocyte-derived macrophage cells (MDM) or an equine dermal cell line (ED). Therefore, these gene-mutated EIAV clones can be produced in large-scale and have been used to prepare a vaccine for safe and effective immunization of horses.

As would be recognized, mutations comprising deletions could be made such that the EIAV contained multiple deletions in genes including the S2 ($\Delta S2$), DU (ΔDU) or both. A gene-mutated EIAV comprising a deletion in the DU(ΔDU) gene was prepared

by deleting a StyI restriction fragment containing 80% of the DU coding sequence, including four of the five conserved amino acid motifs, from the proviral clone designated PV19-2-6A (described by Lichtenstein et al, J. Virol. May 1995, p. 2881-2888 and incorporated herein by reference). It has been demonstrated that the above-described deletion in the *DU* gene does not reduce the ability of this gene-mutated EIAV to replicate in either fetal equine kidney cells (FEK) or in an equine dermal cell line (ED) both considered to be *in vitro* growth. Therefore, it has been demonstrated that this gene-mutated EIAV can be produced in large-scale and vaccine production is possible.

In accord with the invention, it has been found that the *S2* antibodies can be detected in horses with EIAV infections by using immunoassays comprising recombinant, *S2* protein or synthetic *S2* peptides as the capture antigen. Additionally, it has been determined that the presence of the type of virus found in a mammal can be differentiated between the vaccine virus and the wild-type virus by use of gene probes (PCR-based). It has also been determined that the *S2* gene of EIAV is not required for *in vitro* replication in a variety of equine cells including but not limited to fetal equine kidney cells (FEK), equine dermal cell lines (ED) or cultured equine monocytes/macrophages. It has further been determined that the *S2* deletion mutant replicates *in vivo* only at very low levels as compared with the wild-type EIAV (Li, et al, Jan. 2000, J. Virol. Pp 573-579), incorporated herein by reference. By low levels is meant that the virus produces less that $1x10^5$ EIAV particles (as detected by PCR) *in vivo*, preferably less than $1x10^4$. Further, it has been determined that the *S2* protein is not a component of purified EIAV particles and that horses immunized with purified EIAV particles do not produce serum antibodies reactive with *in vitro* synthesized *S2* protein or

peptides. Therefore, even horses vaccinated with purified EIAV particles can be differentiated from wild-type infected horses. These results indicate that the presence of *S2* specific antibody can be used to identify EIAV-infected horses and to distinguish infected horses from those that have been vaccinated with an inactivated whole virus or an attenuated vaccine in which the *S2* gene is mutated so as to make it non-functional. Therefore, it is within the scope of this invention that a diagnostic test can be used to differentiate vaccinated equines from non-vaccinated and/or infected equines by measuring the presence or absence of antibodies to the *S2* protein, to the *DU* protein or to both proteins. Such differentiation can be measured by developing an immunoassay, an antibody-detecting assay (e.g., indirect fluorescent antibody, immunodiffusion, agar diffusion, electrophoresis) or a PCR-based assay known to the art. An example of an immunoassay is an enzyme linked immunosorbant assay (ELISA) that detects and/or quantitates antibodies to specific proteins in serum, blood or tissues. ELISA technology could also be used to detect the presence or absence of virus-associated antigens in the blood, serum or tissues. By virus associated antigens is meant the presence or absence of a gene expression product such as the *S2* or *DU* proteins in the case of the *S2* or *DU* genes, respectively. Additionally, PCR-based assays have been used to measure the presence or absence of genes or gene sequences in the blood, serum or tissues of an equine, thus indicating that a horse had been infected or vaccinated, as the case may be. For this particular embodiment, an ELISA would detect the presence of antibodies to the *S2* or *DU* proteins. If antibodies were present in horses that were tested it would indicate that the horse had been infected with EIAV. Horses that had been vaccinated with a gene-mutated EIAV construct containing a non-functional *S2* gene would not contain *S2*

antibodies in their serum. Horses that had been vaccinated with a gene-mutated EIAV construct containing a non-functional *DU* gene would not contain *DU* antibodies in their serum. Thus, vaccinated horses could be differentiated from infected horses. The PCR-based assays would be used to detect the presence or absence of gene sequences within the horse. For instance, if a horse had been infected with a wild-type EIAV, it would contain the gene sequence for wild-type *S2* or *DU*. However, equines immunized with vaccines comprising a gene-mutated EIAV, particularly one wherein the *S2* or *DU* genes comprised deletions or specific mutations would not contain the gene sequence for wild-type *S2* or *DU* gene products.

As would be recognized from this invention, said mutated (deleted) gene regions could serve as potential points for insertion of foreign genes and that said Δ*S2* or Δ*DU* or a combination thereof, preferably within the Δ*DU*, with a foreign gene insert could be useful as a vector for vaccination against diseases of mammals other than EIA and could serve to protect mammals from a second type of viral, bacterial or parasitic disease. For instance, it would be highly advantageous to incorporate a gene for another important equine disease (e.g., equine influenza, equine herpes virus types 1, 2 or 4, *Streptococcus equi, Rhodococcus equi*) into the gene-mutated EIAV. When such a vaccine is used to vaccinate horses, the horse would not only be protected from disease caused by EIAV but also from disease caused by the other equine disease organism.

Vaccines of the present invention have been either inactivated or administered live. Inactivated vaccines of the present invention comprise treatment of the live virus, attenuated virus, purified virus particles or whole virus particles with agents that inactivate the virus such that it cannot replicate *in vitro* or *in vivo*. Such agents are

selected from the group consisting of formalin, formaldehyde, beta-propriolactone, binary ethyleneimine, ethyleneimine, merthiolate, thimerosal, psoralen and combinations thereof. These agents can be used at concentrations varying from 1 part per billion to 0.5%, depending on the agent. For instance, thimerosal would be used at a concentration of between 1 part per 1,000 and 1 part per billion, preferably between 1 part per 5,000 and 1 part per 100,000. Formalin would be used at a concentration between 0.00001% and 0.5%, preferably between 0.0001% and 0.1%. Ethyleneimine would be used at a concentration between 0.00001M and 0.1M, preferably between 0.0001M and 0.01M. Beta-propiolactone would be used at a concentration similar to that used for ethylenimine.

Vaccines of the present invention may also include adjuvants in order to enhance the immune response. Adjuvants are chemical agents or extracts of microorganisms that induce an enhanced immune response. When accompanied by an antigen, they enhance the immune response produced by the antigen. In the case of EIAV particles, EIAV purified virus particles, EIAV constructs, attenuated EIAV, EIAV (whole virus) or EIAV subunits, adjuvants may be added to enhance the immune response to the vaccine composition to provide improved protection. It is recognized that adjuvants would be used according to the present invention at concentrations varying from 0.1% to 50% v/v, preferably from 1% to 20%.

Although any adjuvant will enhance the immune response and can be used with the vaccine compositions of the present invention, it is within the teaching of the present invention that adjuvants selected from the group consisting of polymer-based, oil-based, block copolymer-based, aluminum salt based, organism-based, lipid-based and aqueous-

based, surfactants are preferred. Non-limiting examples of surfactants useful as adjuvants include hexadecylamine, octadecylamine, lysolecithin, demethyldioactadecyl ammonium bromide, N,N-dioctadecyl-N'-N-bis (2-hydroxyethylpropane diamine), methoxyhexa-decyl-glycerol and pluronic polyols and saponin, Quil A. Non-limiting examples of polyanions or polycations include pyran, diethylaminoethyl (DEAE) dextran, dextran sulfate, polybrene, poly IC, polyacrylic acid, carbopol, ethylene maleic acid, aluminum hydroxide, and aluminum phosphate. Non-limiting examples of peptide adjuvants include muramyl dipeptide, dimethylglycine and tuftsin. Non-limiting examples of other types of adjuvants include oil emulsions, immunomodulators (interleukin-1, interleukin-2 and interferons) or combinations of any of the foregoing adjuvants. A number of acrylic acid polymers and copolymers of acrylic acid and methacrylic acid and styrene have adjuvant activity. Polyvinyl Chemical Industries (Wilmington, MA) provides such polymers under the trade-name NEOCRYL®, BEOCRYL A640, an aqueous acrylic copolymer with styrene. Other useful NEOCRYL products are 520 and 625, and NEOREZ 966. Ethylene maleic acid, produced from ethylene maleic anhydride is a preferred adjuvant. In order to produce ethylene maleic acid, EMA 31 or EMA 91 (Monsanto Co., St. Louis, MO) is prepared in an aqueous solution at a concentration between 0.1 and 10% (w/v), preferably between 0.5 and 5% (w/v). It is used in product at a concentration of 1 to 50% (v/v). More preferably, Carbopol is used as an adjuvant alone or in combination with tweens, spans and oils. Representatives of this type of adjuvant are HAVLOGEN® and SPUR®. These adjuvants are prepared by mixing Carbopol 934P at a concentration between 0.5 and 10% (w/v), preferably between 1 and 5% (w/v), more preferably between 2.0 and 4% (w/v).

Added to the Carbopol can be detergents such as Tween 80 and Span 20, and an oil for producing an emulsion. The oils can be cottonseed, peanut, mineral, or any other type known to be safe for use in animals. The concentrations of the oil ranges from 0.000001% to 10% (v/v), preferably from 0.00001% to 5% (v/v), more preferably from 0.0001% to 1% (v/v). Other commercially-available adjuvants useful for this vaccine include but are not limited to POLYGEN™, a polymer-based low molecular weight, non-particulate copolymer which can form cross-linkages in solution to become a high molecular weight gel (MVP Laboratories, Inc., Ralston, NE) or EMULSIGEN™ or EMULSIGEN™ PLUS, both oil-in-water adjuvants provided by MVP Laboratories, Inc. Organism-based adjuvants are those utilizing whole microorganisms, such as Muramyl Dipeptide, RIBI®, whole Parapox viruses or extracts thereof (also known as Baypamun) and *Corynebacterium acne* extracts. Lipid-based adjuvants include but are not limited to BAY R1005, liposomes and ISCOMS. The most preferred adjuvants of the present invention include HAVLOGEN®, POLYGEN™, BAY R1005, Baypamun and ethylene maleic acid-based. Often, two or more adjuvants can be used to formulate with the EIAV constructs of this invention.

In order to better understand the following Examples, the wild-type EIAV is referred to as the Wyoming isolate or EIAVwyo. This virus is termed a primary isolate and it replicates only in equine monocyte-macrophage cell cultures in which the virus is cytopathic for the infected cells by 7-10 days post infection. Thus, EIAVwyo can be produced only in short-term macrophage cultures to obtain infectious virus in cell supernatants or in experimentally infected horses to obtain infectious plasma (Malmquist et al. 1973, Arch. Virol. 42, p 361-370). Either source of the primary isolate EIAVwyo

can be used to experimentally infect equids and produce classical EIA disease. To obtain a cell-adapted strain of EIAVwyo that is able to replicate in other cell types, the primary EIAVwyo isolate was serially passaged in equine cells to produce a stock of EIAV virus that could be grown on various fibroblastic cells (Malmquist et al 1973, Arch Virol. 42, p 361-370; Parekh et al. 1980 Virology 107:520-525). The cell-adapted EIAVwyo was then grown in fetal equine kidney cell cultures to produce larger amounts of virus and thus used to prepare stocks of the cell-adapted virus designated $EIAV_{PR}$ (Montelaro et al. 1982 J. Virology 42:1029-1038). Inoculation of ponies with the avirulent $EIAV_{PR}$ results in 100% infection but does not produce EIA disease, confirming the attenuated avirulent nature of the $EIAV_{PR}$ strain (Orrego et al., 1982 Am. J. Vet. Res. 43:1556-1560). To obtain a reference strain of EIAV that can be grown in fibroblastic cells and produce disease in experimentally-infected equids, the $EIAV_{PR}$ strain was serially passaged in ponies and isolated in the context of infectious plasma after the third serial passage (Orrego et al. 1982 Am. J. Vet. Res. 43:1556-1560). The *in vivo* serial passage restored virulence to the EIAV, but did not cause it to lose its ability to replicate in cells other than equine macrophages. This virus stock in infectious plasma was designated as host-adapted EIAVwyo. Inoculation of ponies with host-adapted EIAVwyo induced 100% infection and clinical EIA disease (Payne et al. 1987 Virology 161:321-333). In a subsequent set of experiments, a host-adapted EIAVwyo was grown in fetal equine cell culture in the presence of neutralizing immune serum from a pony to generate antigenic neutralization escape mutants by antibody selection that were then biologically cloned to obtain a more homogeneous genomic population (Rwambo et al. 1990 Arch. Virol. 111:275-280). Subsequent stocks of this biologically cloned reference virus produced in

fetal equine kidney cell culture were termed $EIAV_{PV}$ to indicate "pony virulent". Infection of ponies with the biologically cloned $EIAV_{PV}$ results in 100% infection and disease (Hammond et al. 1997 J Virology 71:3840-3852). Since lentiviruses like EIAV exist in nature as complex genomic mixtures termed quasispecies, primary isolates (EIAVwyo) and biological clones ($EIAV_{PV}$) contain a variety of genomic species. To obtain genetically homogenous forms of EIAV, infectious molecular clones were derived from the avirulent $EIAV_{PR}$ (e.g., EIAV 19-2) (Payne et al 1994 J. Gen.Virol. 75:425-429) and pathogenic $EIAV_{PV}$ (Cook et al. 1998 J. Virology 72:1383-1393) reference stocks by standard molecular biology cloning procedures. Inoculation of ponies with infectious virus stocks produced from chimeras with $EIAV_{PR}$ and $EIAV_{PV}$ sequences (e.g., $EIAV_{UK}$) were shown to produce disease in experimentally-infected horses. The infectious molecular clone $EIAV_{UK}$ was the first reported pathogenic molecular clone.

Figure 3b displays the circular map of this infectious molecular clone, $EIAV_{UK}$. In order to provide further information for the following examples, Figure 3c displays the circular map of $EIAV_{UK}\Delta S2$, Figure 3d displays the circular map of $EIAV_{PR}\Delta S2$, and Figure 3e displays the circular map of $EIAV_{UK}\,\Delta DU\Delta S2$.

The invention is further illustrated but is not intended to be limited by the following examples in which all parts and percentages are by weight unless otherwise specified.

EXAMPLE 1

Several different gene-mutated EIAV constructs were prepared according to the methods of Li et al (J. Virol., October 1998, pp 8334-8348) which are incorporated herein

by reference. The basic *S2* gene mutations were designed so as not to disrupt the second exon of Tat 10 base pairs (bp) upstream from the *S2* initiation sequence, the envelope initiator codon just 23 bp downstream from the *S2* start codon sequence, or the putative Rev-response element (RRE) sequences that have been mapped to both the 5' and 3' ends of the *env* gene. A panel of clones with substitutions that introduce one or more premature stop codons (EIAV.2M/X and EIAV.G5/s) or with a deletion of the first 5 nucleotides of the *S2* gene to shift the *S2* ORF (EIAVΔ*S2*) was produced. These are schematically diagrammed in Figures 2a and 2b. The EIAV proviral DNA is shown at the top; the complete deduced amino acid sequence of the putative *S2* protein is shown in single letter amino acid code at the bottom. Stop codons (indicated by arrows) were introduced into various positions in the EIAV *S2* gene to generate the specific mutant virus strains. As would be recognized, all of the constructs would be considered to be non-functional for *S2* and will be referred to herein as Δ*S2*.

S2 mutant constructs were generated using the PCR-Ligation-PCR (PLP) strategy as previously described (Puffer, et. al., 1997 and Li, et. al., 1998). $EIAV_{UK}$ plasmid DNA was used as the template to perform all PCR reactions for generating *S2* mutations except for $EIAV_{UK}$.2M/X.

EIAV.G5/s was generated using $EIAV_{UK}$ as the template by PCR WITH Pfu polymerase (Stratagene) by using mutagenic downstream primer mspe3-5' (SEQ ID NO: 1) with upstream primer s2pst (SEQ ID NO: 2). A second flanking fragment was amplified using mutagenic upstream primers mspe5'-3' (SEQ ID NO: 3) and s2sph (SEQ ID NO: 4).

$EIAV_{UK}\Delta S2$ was similarly generated using $EIAV_{UK}$ as the template by PCR with Pfu polymerase (Stratagene) by using downstream primer S2min/35rev (SEQ ID NO:5) and upstream primer s2pst (SEQ ID NO:2). A second flanking fragment was amplified using mutagenic upstream primer S2min/53for (SEQ ID NO: 6) and s2sph (SEQ ID NO: 4).

Each of these corresponding two adjacent PCR fragments were gel purified, phosphorylated using T4 polynucleotide kinase (Gibco BRL), and ligated by using T4 DNA ligase (Gibco BRL). After inactivation at 65°C for 15 minutes, the ligation reaction was used for a subsequent amplification using upstream primer s2pst (SEQ ID NO: 2) and downstream primer s2sph (SEQ ID No: 4). This product was gel purified, digested with Ncol and Blp1, and then ligated into the Ncol and Blp1 sites of $EIAV_{UK}$.

$EIAV_{UK}$.2M/X, which has its sequence compared with that of $EIAV_{UK}$ in Figure 2b, was generated using the $EIAV_{UK}$G5/s plasmid DNA as a template with downstream primer 2M35/RE (SEQ ID NO: 7) and upstream primer s2pst (SEQ ID NO: 2). A second flanking fragment was amplified using mutagenic upstream primer 2M53/For (SEQ ID NO: 8) and downstream primer s2sph (SEQ ID NO: 4). The final cloning procedure was as described above.

For simplification and because all of the EIAV constructs described are non-functional for *S2* as demonstrated in tissue culture growth studies (as described in EXAMPLE 2), these EIAV constructs have been redesignated $EIAV_{UK}\Delta S2$.

Standard PCR conditions used for the above-described reactions included, one cycle of denaturation at 95°C for 5 min., followed by 35 cycles of denaturation at 95°C

for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The PCR reactions were set up using the following components:

10μL 10X NEB Thermophilic buffer

1.0L μ10 mM deoxynucleotide triphosphates dNTPs

1.0μM forward primer (upstream primer)

1.0μM reverse primer (downstream primer)

10ng template DNA

x μL double distilled water (ddH_2O) (q.s. to 100μL volume)

A 10 μL aliquot was run on an 1.0% agarose gel to make sure the correct size product was amplified. The PCR products were then gel isolated and purified with a Qiaex II gel extraction Kit (150)(Qiagen, Cat. #20021). The Qiaex II protocol is presented below:

1. Cut band from gel and place n a 1.5 mL eppendorf tube.

2. Estimate the volume of agarose gel slice, add 3 volumes of buffer QX1, if the fragment is <4kb, and an additional 2 volumes of ddh_2O if the fragment is >4kb.

3. Vortex the Qiaex II beads and add 10 μL to the agarose slice suspension.

4. Mix well, incubate at 50°C for 5-10 minutes, mixing the tube several times during the incubation period.

5. Centrifuge the sample for 30 seconds and carefully remove the supernatant with a pipette followed by washing the pellet once with 500 μL of buffer QX1.

6. Wash the pellet twice with 500 μL of buffer PE, and air dry pellet 15-30 minutes at room temperature.

7. Resuspend the pellet in 20 μL of ddh_2O, incubate at 55°C for 10 min., spin at full speed for 30 seconds.

8. Pull off supernatant and save to a clean eppendorf tube. Measure the OD at 260nm for the concentration of the recovered fragment on an agarose gel.

9. Add ddH2O as needed to resuspend the pellet.

The two adjacent PCR fragments were individually phosphorylated in the following reaction mixture by using T4 polynucleotide kinase (NEB) prior to ligation. The phosphorylation reaction was set up as follows:

2.0 μL 10X T4 polynucleotide kinase (PNK) buffer (NEB)

2.0 μL 10 mM ATP (NEB)

1.0 μL T4 PNK (NEB)

15 μL gel purified DNA of each of these two adjacent PCR fragments

The reaction was incubated at 37°C for 1 hour. Following inactivation at 65°C for 10 min. the adjacently phosphorylated PCR fragments were then ligated together by using T4 DNA ligase (NEB) under the following conditions:

1.0 μL 10X T4 DNA ligase buffer (NEB)

X μL (50-100 ng) of each of two adjacent PCR fragments

1.0 μL T4 DNA ligase (NEB)

X μL ddH2O (q.s. to 10 μL total volume)

After overnight incubation at 16°C the ligation reaction product was used in a second round PCR reaction to amplify the full-length PCR fragment spanning these two adjacent PCR products. The second round PCR reaction was performed as previously described (see below) with the exception that only upstream primer s2pst (SEQ ID NO: 2) and downstream primer s2sph (SEQ ID NO: 4) were used. Again, a 10 μL aliquot was run on an agarose gel to make sure the correct product was amplified. The full-length PCR fragments were then gel isolated and purified using the Qiaex II kit (see above). The purified full-length PCR fragment, together with $EIAV_{UK}$, were then cut with NcoI (Gibco BRL) and BlpI (Gibco BRL) under the following conditions:

2.0 μL 10X React2 buffer (Gibco BRL)

1.0 μL NcoI (Gibco BRL)

1.0 μL Blp1

X μL full length PCR product (1.0 μg) or $EIAV_{UK}$ (500 ng)

X μL ddh_2O (q.s. to 20 μL total volume)

The above restriction enzyme digestion mixture was incubated at 37°C for 2 hours. Digested DNA fragments from the full-length PCR product and the $EIAV_{UK}$ plasmid were individually gel isolated and purified using a Qiaex II kit as described above. The digested vector $EIAV_{UK}$ and full length PCR fragment were ligated using T4 DNA ligase using the following procedure:

1.0 μL 10X T4 DNA ligase buffer (NEB)

X μL (25-50 ng) digested $EIAV_{UK}$

X μL (200-400 ng) digested full length PCR fragment

1.0 μL T4 DNA ligase (NEB)

X μL ddH2O (q.s. to 10 L total volume)

The ligation reaction was incubated at 16°C overnight and the ligated products were transformed into *Escherichia coli* DH5α (Gibco BRL) by heat shock as described below:

1. Thaw 100 μL of DH5α competent cells and incubate on ice

2. Add 1 μL of ligation mixture to cells, mix gently, and incubate on ice for 30 minutes

3. Heat pulse the tube in a 42° C bath for 45 seconds and incubate on ice for 2 minutes.

4. Add 0.9 mL SOC broth (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 Mm KC1, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose, pH 7.0) and incubate the tubes at 37°C for 1 hour while shaking at 222 rpm.

5. Plate 150 μL of the transformation mixture onto LB-ampicillin (100 μg/mL) plates and incubate at 37°C overnight.

The proviral clones ($EIAV_{UK}$.2M/X, $EIAV_{UK}$G5/s and $EIAV_{UK}\Delta S2$) were then screened by automatically sequencing using a Taq Dye Deoxy Terminator Cycle Sequencer Kit (Applied Biosystems) individually using an internal sense primer S40 (SEQ ID NO: 9) and an internal antisense primer S15 (SEQ ID NO: 10). Following the verification for the mutations in the S2 gene by sequencing, the proviral DNA clones were used for various future studies.

The generation of $EIAV_{UK}\Delta DU\Delta S2$ was based on the modification of the previously studied $EIAV_{PR}\Delta DU$ virus in which the deoxyuridinetri-phosphatase (dUTPase or *DU*) gene segment was deleted by removing a 330-bp Styl restriction fragment (Lichtenstein, et al., 1995). $EIAV_{UK}\Delta DU\Delta S2$ was generated by subcloning into the full-length $EIAV_{UK}\Delta S2$ proviral backbone of a Sstl-Ncol

fragment of $EIAV_{PR}\Delta DU$, which contained a 330-bp deletion in the *DU* gene. $EIAV_{PR}\Delta S2$ was created by subcloning into the full-length $EIAV_{PR}$ proviral backbone of a Ncol-Blp1 fragment of $EIAV_{UK}\Delta S2$, which contained a S2 gene mutation. All of the various constructs discussed above contain a non-functional S2 gene and could be used in vaccines for immunizing horses against diseases caused by EIAV. The constructs are compared with the wild-type EIAV in Figures 1 and 2. Figures 3d and 3e represent the circular maps of $EIAV_{PR}\Delta S2$ and $EIAV_{UK}\Delta DU\Delta S2$.

It is expected that each of the gene-mutated EIAV constructs can be used to prepare either live attenuated or inactivated vaccines for safe and effective immunization of horses from disease caused by EIAV and can be used to differentiate vaccinated horses from infected horses. As indicated previously, it is recognized that inactivation would be produced by adding an appropriate amount of any of the inactivating agents listed previously or others known in the art to be acceptable to lentiviruses. An appropriate amount means the lowest concentration of inactivating agent necessary to inactivate all of the virus particles without damaging the protective antigens (immunogens).

EXAMPLE 2

In order to demonstrate that the gene-mutated EIAV constructs from Example 1 could replicate in large-scale, a tissue culture growth study was conducted. One microgram of proviral clone DNA from each of the constructs

was used to transfect an ED cell line. The ED cell line (ATCC CRL 6288) was grown in 6 well tissue culture plates seeded with between 2 and 4 x 10^5 ED cells per well in 2 mL of the complete growth Minimum Essential Media with Earles salts (EMEM) plus 10% fetal calf serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin (Gibco BRL 15140-122) and 2 mm L-glutamine (Gibco BRL 25030-081). The plates were incubated at 37°C in a CO_2 incubator approximately 16 to 24 hours until the cells were between 50 and 80% confluent. For each transfection, 1 μg of DNA was diluted into 100 μL of OPTI-MEM 1 Reduced Serum Medium (Gibco BRL 18324-012) and 10 μL of Lipofectamine reagent (Gibco BRL 18324-012) was added to 100 μL of OPTI-MEM I Reduced Serum Medium (OPTI-MEM RSM). The two solutions were mixed gently and incubated at room temperature for 30 minutes to allow the DNA-liposome complexes to form. During this time, the ED cell cultures were rinsed once with 2 mL of OPTIMEM I RSM. For each transfection, 0.8 mL of OPTI-MEM I RSM was added to the tube containing the DNA-liposome complexes, the tube was mixed gently and the contents were overlaid onto the rinsed cells. No antibiotics were added during transfection. The DNA-liposome/tissue cultures were incubated for 5 hours at 37°C in a CO_2 incubator. Following incubation, 1 mL of complete growth MEM containing twice the normal concentration of serum was added to the cell culture without removing the transfection mixture. Twenty-four hours following the start of transfection the medium was replaced with fresh complete growth medium (EMEM). Starting the 48 to 72 hours post transfection, aliquots of the tissue culture supernatants were taken at periodic intervals and

analyzed by using a standard reverse transcriptase (RT) assay as a measure of virus production. Supernatants resulting in RT activity were titrated in an infectivity assay based on cell-ELISA readings as described by Lichtenstein et al, 1995. After titer determination, aliquots of each of the virus construct stocks were frozen at -80°C for further evaluation and use. All of the constructs replicated well in both ED cells and in MDM cells producing RT levels of at least 10,000 CPM/10μl which was the normal level of RT activity observed in wild-type $EIAV_{UK}$ (See Figure 4). Further passaging of the transfected cells in larger vessels was accomplished by use of the same techniques as described and serves as the basis for indicating that the constructs prepared in Example 1 could be produced in large-scale and, therefore, could be used to prepare vaccines.

The tissue culture grown virus construct stocks were molecularly characterized by extracting viral RNA and conducting RT-PCR analyses of the *DU* and *S2* genes using 20% glycerol cushion purified virus construct particles. These sequence analyses confirmed the *DU* and/or *S2* gene mutation in their corresponding virus constructs. The RT-PCR technique was also employed to identify recombinant virus construct stocks. Wild-type $EIAV_{UK}$ generated a RT-PCR product of 592 base pairs (bp). In contrast, virus constructs containing the *DU* deletion ($EIAV_{UK}\Delta DU\Delta S2$) resulted in a RT-PCR fragment of 262 bp. S2 gene mutant virus constructs identified as $EIAV_{UK}\Delta DU\Delta S2$, $EIAV_{UK}\Delta S2$ and $EIAV_{PR}\Delta S2$ were also analyzed by the RT-PCR technique. While creating the S2 mutation, a SpeI restriction digestion site was created. RT-PCR and restriction digestion analyses of each of $EIAV_{UK}\Delta S2$, $EIAV_{UK}\Delta DU\Delta S2$, $EIAV_{PR}\Delta S2$ and

$EIAV_{UK}$ virus stocks demonstrated that $EIAV_{UK}$ wild-type virus generated a 539 bp RT-PCR fragment that was resistant to digestion by SpeI. Each of the above-listed S2 virus constructs was susceptible to digestion by SpeI, resulting in cleavage of the 539 bp RT-PCR product into 347 and 192 bp fragments.

EXAMPLE 3

In order to prove that the constructs prepared and grown in large-scale in the previous examples could protect either ponies or horses from disease produced by EIAV, a vaccine was prepared using proviral clone $EIAV_{UK}\Delta S2$. The $EIAV_{UK}\Delta S2$ virus construct was grown in primary fetal equine kidney cells (FEK), filtered through a 0.45μM filter and frozen in aliquots at -80°C. The titers of these virus construct stocks were 10^6 infectious center doses (ICD) per mL, as measured by using an EIAV infectious center assay in FEK cells (Lichtenstein, et al, 1995), incorporated herein by reference.

For these studies, the $EIAV_{UK}\Delta S2$ could have been inactivated, preferably, by using agents such as formalin or binary ethylenimine. Additionally, the virus construct could have been adjuvanted with any of several adjuvants, preferably with a Carbopol-based, polymer-based or lipid-based adjuvant. However, for this experiment, the $EIAV_{UK}\Delta S2$ was used without inactivation or adjuvanting so as to determine whether it would replicate *in vivo* as well as it replicated *in vitro*. Thus, this example describes the use of an attenuated live vaccine comprising $EIAV_{UK}\Delta S2$.

The $EIAV_{UK}\Delta S2$ was tested for its ability to protect equines (ponies in this experiment) against an intravenous challenge with pathogenic $EIAV_{PV}$, a heterologous EIAV. The results of this vaccination/challenge study are shown in Table 1. Each of three ponies was vaccinated once with 1.0 mL of the undiluted $EIAV_{UK}\Delta S2$ virus construct stock. Six months after vaccination all 3 vaccinated ponies were challenged intravenously with 300 median equine infectious doses (MEID) of pathogenic $EIAV_{PV}$. All ponies were clinically monitored and maintained in isolation as described by Hammond, et al. (Virology vol: 254, p 37-49). Rectal temperatures and clinical status were recorded daily. Samples of serum, plasma and whole blood were collected from each pony at predetermined intervals. Plasma samples were stored at -80°C until further processed for semi-quantitative viral RNA analyses or identification of the presence of wild-type challenge virus, and serum samples were stored similarly until testing for quantitative and qualitative serological assays could be performed. Whole blood samples were appropriately fractionated for enumeration of platelets or experimentation with PBMCs. Results are shown in Table 1.

During the course of the 6-month immunization, no clinical signs were observed in the vaccinated ponies. This indicates that $EIAV_{UK}\Delta S2$ is avirulent for ponies. To assess virus replication following vaccination, the level of viral RNA in plasma was determined by using a semi-quantitative RT-PCR assay (Li et al, J. Virol, Jan 2000, p. 573-579). EIAV RNA was detected in the plasma of all immunized animals on day 6 after vaccination and unpredictable viremia episodes were observed throughout the course of vaccination. However, the plasma viral

RNA levels observed in the vaccinated ponies were 10-6000 fold lower than the levels measured in ponies previously infected with the parental $EIAV_{UK}$ virus over a six month observation period. This finding indicates that the $EIAV_{UK}\Delta S2$ virus construct is highly attenuated due to the absence of the S2 gene and therefore, the vaccine was safe in equids even in live form.

At 6 months post vaccination, the ponies were challenged intravenously with 300 median equid infectious doses of pathogenic $EIAV_{PV}$. Following challenge of non-vaccinated ponies, clinical signs of EIA are normally apparent in about 16-19 days. Concurrent with the initial EIA-related fever is a rapid decline in quantity of platelets circulating in the blood. In some cases, control ponies recrudesce with more severe clinical manifestations of high fever (>103°F) and extremely low blood platelet counts of <105,000/μL of blood. Following challenge in these ponies, clinical signs of EIA were not evident at all throughout the 3 months observation period indicating that these ponies were successfully protected from disease.

In order to determine whether the ponies were protected from infection (sterile protection), genetic analyses of viral RNA present in the plasma was performed by a nested RT-PCR technique used in combination with differential restriction digestion of RT-PCR product for the differentiation of vaccine strain $EIAV_{UK}\Delta S2$ and wild-type challenge strain $EIAV_{PV}$. All of the vaccinated/challenged ponies demonstrated only the presence of the attenuated

$EIAV_{UK}\Delta S2$ virus construct. Therefore, all (100%) were protected from infection by wild-type EIAV.

Table 1 Summary of Results of Pony Vaccination/Challenge Study

Group	Pony No.	Febrile Episode Post Challenge	Abnormal Blood Count Post Challenge	Protection From Disease[a]	PCR Detection of Challenge strain $EIAV_{PR}$	Protection From Infection[b]
$EIAV_{UK}\Delta S2$	94-11	NONE	NONE	YES	NEGATIVE	YES
	676	NONE	NONE	YES	NEGATIVE	YES
	674	NONE	NONE	YES	NEGATIVE	YES

[a]Animals protected from clinical disease did not demonstrate any progression to clinical disease including temperature and platelet count

[b]Animals protected from infection did not demonstrate any level of expression of wild-type challenge virus at the plasma samples of vaccinated horses by a semi-quantitative RT-PCR Li et al, J. Virol, Jan 2000)

EXAMPLE 4

A vaccination/challenge study similar to that described in Example 3 was conducted with horses using the multiple low dose challenge. This study was conducted in order to demonstrate equivalency between vaccination of ponies or horses as well as demonstration that the multiple low dose EIA equine challenge can serve as a successful model for EIAV infection. The method of Example 2 was used for growth of the $EIAV_{UK}\Delta S2$ construct. Six horses were vaccinated with 1.0 mL of the virus construct. One horse was left unvaccinated to serve as a Control horse. In this study, horses were challenged using the multiple low dose challenge method developed and described in U.S. Pat. No. 6,528,250, incorporated herein by reference. In the multiple low dose EIA equine challenge, each horse received three intravenous inoculations of 10 median horse infective doses (MHID) of $EIAV_{PV}$ at two-day intervals. After challenge, the horses were monitored for clinical signs of EIA for about 3 months post challenge. Results of the horse challenge are shown in Table 2.

Table 2 Summary of Results of Horse Vaccination/Challenge Study

Group	Horse No.	Febrile Episode Post Challenge	Abnormal Blood Count Post Challenge	Protection From Disease	PCR Detection of Challenge strain $EIAV_{PR}$	Protection From Infection
$EIAV_{UK}\Delta S2$	60	NONE	NONE	YES	NEGATIVE	YES
	971	NONE	NONE	YES	NEGATIVE	YES
	615	NONE	NONE	YES	NEGATIVE	YES
	9791	NONE	NONE	YES	NEGATIVE	YES
	9809	NONE	NONE	YES	NEGATIVE	YES
	9812	NONE	NONE	YES	NEGATIVE	YES
CONTROL	880	YES	YES	NO	POSITIVE	NO

These data indicate that a vaccine that protects from both disease and infection in ponies and/or horses produced by EIAV can be prepared from $EIAV_{UK}\Delta S2$. Equines vaccinated with the attenuated $EIAV_{UK}\Delta S2$ construct can be differentiated from infected equines based on the lack of antibody to the S2 protein in vaccinated animals. Such lack of antibody can be determined by any immunological assay known to the art that would demonstrate the presence of S2 antibodies in the blood or serum of infected ponies or horses and the lack of such antibodies in vaccinated ponies or horses. Alternatively, a PCR-based assay known to the art, could be used to detect the presence of the S2 gene sequence in infected horses as compared to the lack of this gene sequence in vaccinated horses. The horse experiment demonstrates that the multiple low dose EIA equine challenge model is effective in both reproducing EIA and in demonstrating that horses can be protected from by a vaccine prepared according to the present invention.

EXAMPLE 5

Live attenuated vaccines were also prepared from the EIAV constructs designated $EIAV_{UK}\Delta DU\Delta S2$ and $EIAV_{PR}\Delta S2$ according to the methods described in Example 3. Two groups of horses were each inoculated intramuscularly two times (at monthly intervals) with the respective attenuated vaccine. Each vaccine contained approximately 10^5 infectious-center doses (ICD) in a 1.0 mL dose. Inoculated horses were monitored daily for any clinical signs of EIA post vaccination. Blood samples were taken at weekly intervals for evaluation of vaccine virus replication and for EIA-specific immune responses. At 6 months post vaccination, all 16 vaccinated horses and 2 non-vaccinated control horses were challenged with the multiple low dose EIA equine challenge with

$EIAV_{PV}$ pathogenic virus stock as described previously. The multiple low dose challenge involved inoculating each horse three times with 10 median horse infective doses (MHID) at two-day intervals. The horses were monitored for clinical signs of EIA, for seroconversion in commercial diagnostic assays for p26 and for infection with the challenge virus using RT-PCR for about 3 months post challenge as in EXAMPLE 3. Table 3 summarizes the results of this study.

Seven of eight (88%) of the $EIAV_{UK}\Delta DU\Delta S2$ vaccinated horses remained asymptomatic post challenge, while six of eight (75%) of the $EIAV_{PR}\Delta S2$ vaccinates were protected from disease post challenge. These clinical data indicate that the vaccines were effective in preventing disease post challenge exposure to a pathogenic $EIAV_{PV}$. However, these vaccines were not as effective as the vaccine tested in Example 3. It is proposed that the reduced protection results from these constructs either being prepared from an avirulent clone of EIA ($EIAV_{PR}$) or a double deletion mutant of the virulent parent clone ($EIAV_{UK}\Delta DU\Delta S2$). It is proposed that addition of an adjuvant to the vaccines of this example would improve their immunogenicity (ability to protect horses from disease) and produce a vaccine that is more protective for disease caused by EIA virus.

Surprisingly, not all of the vaccinated horses seroconverted to p26 as measured by testing for positive antibody status using the Coggins Test. This indicates that a normal p26 assay could be run on vaccinated horses. In order to use this vaccine for commercial purposes, any vaccinated equines that were found to be Coggins Test positive could be confirmed with a test for antibodies for the S2 expression product. If S2 antibodies were

present, it would be confirmed that the horses had been infected with a field strain of EIAV (wild-type) and not the EIAV vaccine of the present invention.

It is apparent that a vaccine composition for effectively and safely immunizing equines from disease caused by EIAV can be produced and that vaccinated equines can be differentiated from infected equines using the standard Coggins test for antibodies to p26 in addition to a test for antibodies to S2 protein or detection of a gene sequence associated with the S2 gene. Antibodies to both proteins as well as the S2 gene sequence are absent in vaccinated and uninfected equines but present in infected equines. Additionally, the absence of antibodies to the DU protein and/or the DU gene sequence can serve as a differential diagnostic test for equids vaccinated with the $EIAV_{UK}\Delta DU\Delta S2$.

It is expected that the attenuated vaccines described in this example were more attenuated than desired. In order to increase their immunogenicity (ability to protect from disease and infection) an adjuvant can be added to the attenuated vaccine or the attenuated viruses can be inactivated as described previously, adjuvanted and administered as repeat doses (2 to 3) for the vaccination series. It is expected that such a modification would protect completely from disease and infection.

Table 3 Summary of Attenuated EIAV Vaccine Trail

Group	Horse	Febrile Episode	RNA $>10^5$	$EIAV_{PV}$ Positive	P26 ANTIBODY Positive
$EIAV_{PR}\Delta S2$	811	X		X	X
	9705			X	X
	9704			X	
	9717			X	X
	9615	X	X	X	X
	9613		X	X	X
	9716			X	X
	9712			X	X
$EIAV_{UK}\Delta DU\Delta S2$	9708			X	X
	9706			X	X
	673		X	X	X
	677				X
	9711				X
	666	X	X	X	X
	711				
	699				
Control	9714	X	X	X	X
	9720	X	X	X	X

EXAMPLE 6

In order to determine whether a vaccine comprising only a *DU* gene-mutated EIAV would be safe and effective in equines, a *DU* gene-mutated EIAV construct was prepared and tested in a horse vaccination/challenge model for EIAV as described in Examples 3 and 4. The *DU* coding region of EIAV is located within the *pol* open reading frame, positioned between the RT and integrase (IN) genes (See Figure 5). It is specifically codes for a dUTPase, an enzyme to convert dUTP to dUMP + pp_1. The predicted amino acid sequence of the EIAV *DU* protein shows a high degree of homology to the dUTPases of other nonprimate lentiviruses and to the human, yeast and *E. coli* enzymes as well. Five conserved amino acid motifs present in all known dUTPase proteins have been recognized and at least one of these motifs has been suggested to be functionally important. Motif 3 contains a highly conserved tyrosine residue, which has been suggested to be involved in catalysis. To construct an EIAV mutant that would be deficient in dUTPase activity, a Styl restriction fragment containing 80% of the *DU* coding sequence, including four of the five conserved amino acid motifs, was deleted from the provirus clone $EIAV_{PR}$. The deletion left intact the *pol* open reading frame and both protease-processing sites present on either side of the *DU* gene. More specifically, to construct the $EIAV_{PR}\Delta DU$ that is deficient in dUTPase activity, a 330 bp restriction fragment from a Kpnl-Pstl *pol* subclone of the proviral clone $EIAV_{PR}$, was deleted. This deleted segment was then subcloned back into a full-length provirus backbone as an Sstl-Ncol fragment to create the mutant provirus clone $EIAV_{PR}\Delta DU$ (see Figure 5). Figure 5 shows the genomic organization of EIAV and the location of the *DU* gene. The position of the two Styl sites used to create the deletion are also shown. The

stippled bar represents the approximate positions of five conserved amino acid motifs present in all known DUTPase genes. Nucleotide and amino acid sequences of *DU* flanking the two Styl sites are shown at the bottom. The leucine residue is the first amino acid of mature *DU* protein. A pol subclone containing the *DU* gene was digested with Styl, and the resulting 5' termini were filled in with T4 DNA polymerase and ligated to generate the sequence shown by the arrow. The deleted ~gene was then inserted back into a full-length proviral clone.

The mutant produced as described, was tested for its ability to replicate *in vitro*, a requirement for large-scale vaccine production. FEK cells and the ED cell line were transfected with the $EIAV_{PR}\Delta DU$ as described previously in Example 2. It was determined that the RT activity was equal to that of wild-type $EIAV_{UK}$. However, when equine macrophage cultures were transfected with this construct at a multiplicity of infection (MOI) of 0.01, very little replication (as measured by RT activity) was noted. This suggests that such a construct would replicate poorly if at all in horses. The tissue culture grown proviral construct was confirmed to be $EIAV_{PR}\Delta DU$ by RT-PCR. These experiments determined that $EIAV_{PR}\Delta DU$ could be produced *in vitro* in large scale in either FEK or ED cells.

In order to determine whether a vaccine could be prepared and whether such a vaccine would protect horses from disease and/or infection, the ED cell line was transfected and a large quantity of $EIAV_{PR}\Delta DU$ was produced. In this study, the proviral construct was inactivated by addition of 0.1% formalin and adjuvanted with a polymer-based adjuvant, specifically with a Carbopol-based adjuvant designated HAVLOGEN®. Two vaccines were formulated. One contained 50μg/dose (1.0 mL) while the second

contained 10μg/dose Each of three horses received 3 doses of 50μg/dose vaccine and each of three horses received 3 doses of 10μg/dose vaccine . The interval between vaccinations was one month. Three additional horses were left unvaccinated and served as negative controls. Nine weeks post final vaccination, all horses were challenged with a multiple low dose challenge using $EIAV_{PR}$, a heterologous strain. This constituted administering 10 MHIDs three times over a 7 day period (days 0, 2 and 5). Horses were monitored for temperature, platelet count, plasma viremia and seroconversion for 7 weeks post challenge. Results of this vaccination/challenge study are shown in Table 4.

Table 4 Summary of Results of the Vaccination/Challenge Study using an inactivated, Adjuvanted *DU* gene-mutated EIAV Vaccine

Group	Horse	Febrile Episode	RNA $>10^5$	$EIAV_{PV}$ Positive	P26 ANTIBODY Positive
$EIAV_{PR}\Delta DU$ 50μg/dose					
	710	None	Neg	Neg	X
	682	None	Neg	Neg	X
	95-03	None	Neg	Neg	X
$EIAV_{PR}\Delta DU$ 10μg/dose					X
	787	X	X	X	X
	785	X	X	X	X
	724	None	Neg	Neg	Neg
Controls					
	96-08	X	X	X	X
	827	X	X	X	X
	746	X	X	X	X

It is noted from Table 5 that all three horses receiving a 50μg/dose of inactivated, adjuvanted vaccine were protected from both disease and infection. These horses demonstrated no clinical signs of disease and did not demonstrate the presence of challenge virus (viremia) as measured by RT-PCR. Even a dose of only 10μg was able to protect 1 of 3 horses from both disease and infection. All control horses demonstrated both disease and infection typical of full-blown EIA. This is an extraordinary result, especially since the challenge virus that was administered was heterologous, not homologous to the vaccine constructs. These data prove that the teachings of the present invention can be used to prepare a completely protective vaccine. It also proves that inactivation and adjuvanting do not decrease the immunogenicity of the EIAV vaccines of the present invention.

EXAMPLE 7

To address concerns about possible reversion of stop codons, even redundant stop codons, a true nucleotide deletion in the region of the S2 gene that lies outside of the envelope open reading frame was also generated. The $EIAV_{UK}\Delta S2$ construct described in EXAMPLE 1 was utilized as the proviral backbone of the true deletion proviral clones. The region to be deleted was limited due to the overlap of the S2 open reading frame with the envelope reading frame. The primary site of deletion was to be no less than the ATG start codon of the S2 gene and the three nucleotides upstream of the ATG as they could possibly create an alternative ATG start codon in the absence of the original ATG start codon. (see Figure 6) The deletions were made very deliberately in specific regions upstream and outside of the envelope open reading frame in order to not to affect viral envelope expression. Furthermore, possible deletion regions were chosen so as not to

disturb the TAT stop codon (second exon) or splice donor site upstream of the S2 start codon; these deletions were also generated as additions to the two stop codons and outside of and not disrupting the molecular marker Spe I digestion site within the first stop codon (G^5). (see Figure 6) Several different length deletions were studied to determine which would most reflect the parental $EIAV_{UK}\Delta S2$ replication properties. To generate the nucleotide deletions, $EIAV_{UK}\Delta S2$ was used as template for PCR reactions again in which two adjacent fragments were amplified spanning the S2 open reading frame outside (and not including) the base pairs to be deleted. The first two deletions attempted were a 14 base pair deletion (primers: S2VT6p.REV: ATA TCA AAC CTT ATA ACA AAT ATT GAG GTT G (SEQ ID NO 11), S2vt6p.for: TAC TAG TGT AAA GGG GTA ACA TGG TCA (SEQ ID NO 12)) and a 25 base pair deletion (primers, S212BP.FOR: TAC TAG TAC ATG GTC AGC ATC GCA TTC TAC GGG GTG (SEQ ID NO 13), S2VT5mp.REV: AAA CCT TAT AAC AAA TAT TGA GGT TG (SEQ ID NO 14)). (see Figure 7) The standard PCR conditions detailed in EXAMPLE 1 were employed again as was the ligation procedure prior to the second round of PCR. The PCR products were also gel purified as described in EXAMPLE 1. The resultant two ligated-PCR products of the individual primer pairs (14bp deletion and 25bp deletion) were used independently as templates for the second round of PCR employing the outer primer pair of each deletion primer pair. The final PCR product was digested as previously described in EXAMPLE 1 and cloned back into $EIAV_{UK}\Delta S2$ also previously digested with NcoI and BlpI, again detailed in EXAMPLE 1. All plasmid clones were sequenced as illustrated in EXAMPLE 1 to verify introduced mutations and deletions and to ensure the integrity of the PCR-amplified sequence.

Following verification of the sequence the proviral clones were transfected into equine dermal (ED) cells, in a manner equivalent to that described in EXAMPLE 2, two clones for each deletion, to ascertain how the deletions affected their *in vitro* replication properties. (see Figure 8)

It appeared from these results that the larger the nucleotide deletion, the greater the replication of the virus was attenuated. The 14 base pair deletion clones displayed replication at approximately 1 $\log_{10}$ lower than the $EIAV_{UK}\Delta S2$ dual-stop codon clone. Therefore it was determined that the next deletion mutants would be of smaller nucleotide values.

The next two clones generated were 9 and 6 base pair deletions generated using a similar, but different method of cloning. Instead of using the PCR-Ligation-PCR method which has been described in detail in EXAMPLE 1, overlapping primer PCR cloning was utilized for these smaller deletions. In general, the method involves using two rounds of amplification. The first round uses primer sets which bind directly upstream or downstream of the nucleotide region to be deleted. The boundary primer from each of the two primer sets contains several nucleotides which overlap with the other primer set nucleotides that allow for amplification of the two first-round amplicons into a single amplified region (minus the deleted region) using the outer primers from each set of primers from the first round of amplification, in the second round of amplification.

The 9 base pair deletion primer pairs consisted of the following nucleotide fragments: Upstream of the deletion, DS2.5'F: AGG GAA AGT ATG GGA GGA CAG ACA CC (SEQ ID NO 15), and 9BP5'R: GTA TAC TCA AAC CTT ATA ACA AAT ATT GAG

GTT (SEQ ID NO 16); Downstream of the deletion, DS2.3'F9: GTT TGA GTA TAC TAG TGT AAA GGG GTA AC (SEQ ID NO 17), and DS2.3'R: CCA AAG TAT TCC TCC AGT TCC TGC (SEQ ID NO 18). The 6 base pair deletion primer pairs consisted of the following nucleotide fragments: Upstream of the deletion, DS2.5'F: AGG GAA AGT ATG GGA GGA CAG ACA CC (SEQ ID NO 19), and 6BP5': TAC TCC TCA AAC CTT ATA ACA AAT ATT GAG GTT (SEQ ID NO 20); Downstream of the deletion, DS2.3'F6: GTT TGA GGA GTA TAC TAG TGT AAA GGG G (SEQ ID NO 21), and DS2.3'R: CCA AAG TAT TCC TCC AGT TCC TGC (SEQ ID NO 22). (see Figure 9)

Standard PCR conditions used for the above-described reactions included, one cycle of denaturation at 95°C for 3 min., followed by 35 cycles of: denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 30 seconds. The PCR reactions were set up using the following components:

10μL 10X NEB Thermophilic buffer

1.0L μ10 mM deoxynucleotide triphosphates dNTPs

1.0μM forward primer (upstream primer)

1.0μM reverse primer (downstream primer)

10ng template DNA

x μL double distilled water (ddH_2O) (q.s. to 100μL volume)

A 10 μL aliquot was run on an 1.0% agarose gel to make sure the correct size product was amplified and gel purified as detailed in EXAMPLE 1. The resultant two PCR products (9bp deletion and 6bp deletion) of the individual primer pairs were used independently as templates for second rounds of PCR employing the outer primer pair of each deletion primer pair. Standard PCR conditions used for the reactions included, one cycle of denaturation at 95°C for 1 min., followed by 35 cycles of: denaturation at 94°C for 30 seconds, annealing at 68°C for 30 seconds and extension at 72°C for 30 seconds. The PCR reactions were set up using the following components:

10μL 10X NEB Thermophilic buffer

1.0L μ10 mM deoxynucleotide triphosphates dNTPs

1.0μM forward primer (upstream primer)

1.0μM reverse primer (downstream primer)

10ng template DNA

x μL double distilled water (ddH_2O) (q.s. to 100μL volume)

A 10 μL aliquot was run on a 1.0% agarose gel to make sure the correct size product was amplified and gel purified as illustrated in EXAMPLE 1. The final PCR product was digested as previously described in EXAMPLE 1 and cloned (as illustrated in EXAMPLES 1) back into $EIAV_{UK}\Delta S2$ also previously digested with NcoI and BlpI. All plasmid clones were sequenced (detailed in EXAMPLE 1) to verify introduced mutations and deletions and to ensure the integrity of the PCR-amplified sequence. The proviral

clones were then transfected into ED cells, in a manner equivalent to that described in EXAMPLE 2, to ascertain how the deletions affected their *in vitro* replication properties.(see Figure 10)

Surprisingly, the 6 base pair deletion (D6) did not show optimal replication properties where the 9 base pair deletion (D9) did. The D9 provirus reflected similar replication properties to the $EIAV_{UK}\Delta S2$ and the wildtype $EIAV_{UK}$ constructs, where the D6 displayed almost 2 logs lower activity as compared to the other 3 virus constructs. This was in direct contrast to the observations from the first two deletion clones, wherein the results indicated that the smaller nucleotide deletions displayed enhanced replication properties as compared to larger deletions. This was however a transfection of proviral DNA in equine dermal cells, not an infection of the primary target cell, equine macrophage. Hence to test if this was an experimental phenomenon or an actual characterization of the deletion mutants, equine macrophage were infected with supernatants from 15 days post transfection. Suprenatants from all transfections displayed in Figure 10 (see Figure 11) were tested for reverse transcriptase activity in a standard reverse transcription assay. Volumes of infectious supernatant were overlaid on equine monocyte derived macrophage (1×10^5 in 48 well plates) which contained equivalent RT values. These infections were incubated for 2 hours at 37°C/6% CO_2. The infectious supernatants were then removed and replaced with 0.5 mL MEM alpha (GIBCO). Aliquots were taken for RT analysis as was done for transfections.

Once again, the D6 clone demonstrated a lower replication curve and the D9 clone emulated the $EIAV_{UK}\Delta S2$ virus. Therefore, the D9 clone became the primary candidate for a live attenuated EIAV vaccine.

Although the invention has been described in detail in the foregoing, for the purpose of illustration it is to be understood that such detail is solely for that purpose and that variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention except as it may be limited by the claims. Furthermore, all patents and printed publications mentioned herein are herby incorporated by reference.